Seasonal timing in a warming world

Salis, Lucia

Chapter 5

Photoperiod at the larval stage sets the timing of entire annual program in the winter moth

Lucia Salis, Erik van den Hoorn, Domien G.M. Beersma, Roelof A. Hut & Marcel E. Visser

Submitted

Abstract

Organisms need to synchronize their life-cycles to the annual variation in environmental conditions to maximize their fitness. A key question is whether a phenological advancement in one life-cycle stage is carried over to the subsequent stages, or that the subsequent stages are not affected as the underlying circannual clock is not shifted. In our long-term study (1994-2014) on the winter moth, timing of egg-hatching strongly varies between years. But there is little inter-annual variation in timing of adult eclosion. To investigate how the timing of adult eclosion remains uncoupled from the timing of egg-hatching, we kept winter moth larvae under photoperiodic regimes that mimicked the natural schedule but started on an earlier or later calendar date than the real date. The photoperiodic schedule experienced during the larval stage did not alter the duration of larval development but it affected the subsequent pupal and egg developmental time. A photoperiodic schedule that resembled an earlier calendar date, led to a longer annual cycle, with later adult eclosion and later egg-hatching. These findings show that winter moths can use photoperiodic information acquired during the larval stage to set the timing of their annual program by adjusting the duration of subsequent life-cycle stages.

Introduction

In temperate ecosystems organisms need to cope with annual fluctuations in dark-light regime, temperature, rainfall and humidity. Due to these abiotic changes and the associated changes in the biotic environment, suitable conditions to reproduce, grow or mate are often restricted to a limited period in the year. To adequately exploit these suitable conditions species need to synchronize their life-cycle with environmental changes. When organisms fail to synchronize with the environmental conditions at one or more life-stages their fitness will be reduced (Jordano *et al.* 1994; Durant *et al.* 2005; van Asch *et al.* 2007b). In most species, conditions experienced at one stage of the life-cycle can influence subsequent life-cycle stages (i.e. carry-over effects). Carry-over effects are widespread in nature and likely to cause large variation in individual fitness (Harrison *et al.* 2011). These carry-over effects are therefore of key importance when studying the relationship between an organism's phenology in relation to variation in the annual environmental cycle, but are often difficult to study as individuals need to be followed across their entire life-cycle. A phenological advance (or delay) of the development at one life-stage may have positive effects on current fitness but could reduce future fitness. Carry-over effects will lead to a phenological mismatch when cues at one stage are not reliable to predict the optimal timing of subsequent life-stages. In these cases, organisms need to offset temporal shifts induced during a previous stage to reconcile their life-cycles with the annual environmental variation in the next stage.

Environmental variables used to regulate seasonal timing can be highly species-specific, but photoperiod and temperature are widespread phenological cues used by most species. Photoperiod provides a precise and reliable cue as its variation at a given latitude is invariant from year-to-year. For most organisms, photoperiod is a clear signal that initiates a cascade of physiological and behavioural modifications that can even be effective many generations later (Bradshaw *et al.* 2007). Migration in birds (Dawson *et al.* 2001; Dawson 2002) and diapause (i.e. state of reduced metabolic activity which occurs when conditions are not favourable) in insects (Tauber *et al.* 1976; Mousseau *et al.* 1991; Denlinger 2002) are some examples demonstrating the impact of photoperiod on phenology. Many species also use the annual variation in temperature as cue to forecast upcoming environmental conditions, often in combination with photoperiod. Moreover, temperature is not only used as cue, but in most ectotherms species it also has a strong direct effect on developmental rate and thus phenology (Bale *et al.* 2002).

In the winter moth (*Operophtera brumata*, L.), egg-development is highly temperature dependent (Embree 1970; Holliday 1985; Kimberling *et al.* 1988; Visser *et al.* 2001). The winter moth is an herbivorous insect with an annual cycle (Figure 1): adults emerge in winter from their pupae buried in the soil. After mating, females

lay eggs in clutches on oak trees (*Quercus robur*) and the eggs develop throughout winter. In this species, timing of egg-hatching ranges from early to late spring, depending on the ambient temperatures experienced during egg development. In warm springs, temperatures lead to early egg-hatching. After hatching the larvae feed on young oak foliage until pupation in the soil and the life cycle is started again. Synchrony of winter moth egg-hatching with the bud bursting of its host plant is essential for survival and growth of the larvae. If the eggs hatch too early relative to the oak bud burst, the newly hatched larvae starve to death within few days as there is no food available. On the other hand, if the eggs hatch too late, the quality of the oak leaves is lower due to the increased tannin concentration. Timing of egg-hatching thereby affects pupal weight and thereafter fecundity at the adult stage (van Asch *et al.* 2007a).

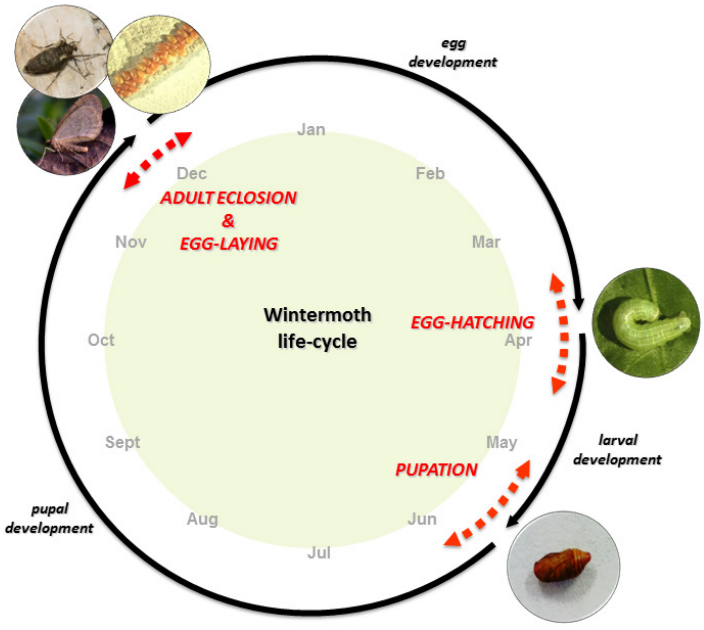

Figure 1 Winter moth life-cycle. Adult moths eclose in winter and lay eggs on their host plant. Eggs develop throughout winter and hatch in spring. Larvae feed on buds and fresh leaves till pupation. Pupae are buried in the soil at about 10 cm depth and eclose as adults in winter. Thick arrows (in black) indicate the duration of each life-cycle stage; dashed arrows (in red) indicate the temporal variability of each event.

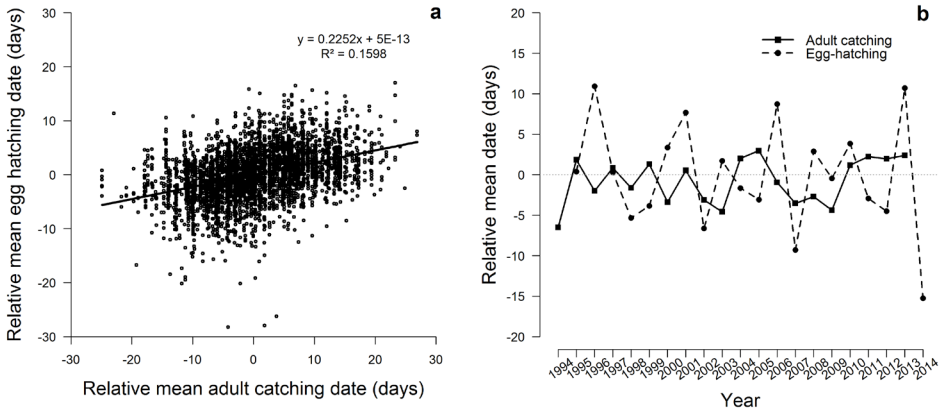

Figure 2 a) Relationship between adult catching date and egg-hatching date relative to the yearly mean adult catching and egg-hatching date respectively. Each data point represents an individual moth and its egg clutch (long-term field data; 1994-2014). b) Fluctuations of egg-hatching and adult catching date. Plotted values represent the differences between the yearly mean and the overall mean of the period studied (1994-2014)).

In the field, our long-term data show that egg-hatching indeed varies considerably from year to year (Figure 2b; $SD_{egg\text{-}hatching}$=6.7), due to differences in temperature from year to year. In addition to this temperature-induced plasticity in timing of egg-hatching, timing of egg-hatching is also correlated with the time at which the eggs were laid, i.e. the time when their mother was active (VanDongen *et al.* 1997). Our long-term data show that, irrespective of temperatures, eggs of moths caught later in the years (i.e. late December) tend to hatch later next spring (Figure 2a). However, at the timing of adult eclosion environmental cues have very little predictive power to forecast the temperature conditions in next spring and thus the optimal timing of egg-hatching. As spring temperatures vary each year, carrying over the phenological advance (or delay) gained during egg-development to the following stages till adult eclosion is maladaptive and can lead to a mismatch at the next generation. As a consequence, the best "decision" at the adult stage is to eclose at a fixed date and therefore reset the life-cycle with the calendar date. In fact, in the field mean adult catching date (considered here as proxy for timing of adult eclosion as adults only live for a few days) varies little from year-to-year (Figure 2b; $SD_{adult\ eclosion}$=2.8), despite the large variation in annual mean egg-hatching date. This shows that winter moths do compensate the temperature-induced shifts at egg-hatching in the course of their life-cycle.

It is unclear how adult eclosion date is synchronized with a fixed calendar date. Pupae develop in the soil, at about 10-15 cm depth, where photoperiodic cues are absent and the annual variation in temperature is mitigated. Thus, it is more likely that the winter moth life cycle is reset during larval development when the animals are exposed to photoperiod and daily temperature changes. Therefore, we aimed to unravel how timing of adult eclosion remains linked to the calendar date while being uncoupled from the egg-hatching date. In our experiment, we kept winter moth larvae from egg-hatching till pupation under five artificial photoperiodic regimes that mimicked natural photoperiods starting on earlier or later calendar dates than the actual current date. Subsequently, we recorded the developmental time of each stage of the life-cycle and the timing of transition from one stage to another for the entire annual cycle including the timing of egg-hatching of the next generation.

Materials and methods

Collection of long-term data

To collect long-term data on timing of winter moth adult eclosion and egg-hatching we caught adult winter moths in four forests in the Netherlands (Doorwerth, Hoge Veluwe, Warnsborn and Oosterhout) from 1994 till 2014. Each autumn we placed funnel traps on oak trees (*Quercus robur*) in each forest at several sites. From late October till early January traps were checked every other day and, on the day of capture, moths were brought to the laboratory, weighed and placed in transparent vials. Each vial contained a female and, if available, a male caught on the same date in the same trap. All females were provided with a piece of tissue paper to lay their eggs on and were kept in an outdoor insectarium. After death, adult moths were removed; the paper tissues with eggs were transferred to Petri dishes and kept in an outdoor insectarium until hatching. Egg-hatching was checked three times a week and median egg-hatching date was calculated as the date at which 50% of the clutch had hatched.

Origin of the experimental animals

For the laboratory experiment we collected adult winter moths as described in the previous section (a) in one forest (Oosterhout; 51°52’22.30»N, 5°50’21.77”E) from 30th November to 5th December 2012. The egg clutches were kept in the outdoor insectarium until the start of the experiment. From each clutch, three newly hatched larvae were placed in individual Petri dishes and allocated to each photoperiodic treatment, following a split-brood design, thus creating groups with the same genetic composition. In total three replicas from 40 egg clutches were used for the

experiment. Because mortality is high during the first instar, each single replica consisted of three larvae placed in the same Petri dish, to guarantee three replicas per clutch in each treatment. After ten days from the start of the experiment, if more than one larva survived, the other larvae were randomly removed so that finally each replica consisted of one single larva per petri-dish.

Experimental design

The experiment was conducted between the 1st of May 2013 and mid June 2014. At the start of the experiment larvae were allocated to five artificial photoperiodic treatments in climate-controlled cabinets (Sanyo MIR552 / MIR553). The five photoperiodic treatments resembled the change in day length as under natural conditions at the same latitude. Winter moth larvae were exposed to photoperiodic treatments that mimicked change in day length starting both on earlier and later calendar dates than the control treatment: 1st April (very early photoperiod); 15th April (early photoperiod); 15th May (late photoperiod) and 1st June (very late photoperiod). The mimicked starting date of the control treatment corresponded to the starting date of the experiment: 1st May (control photoperiod). In all treatments the schedule was adjusted every day following the natural progressing change in day length as from the starting date (Figure 3). All treatments started on the same day. For simplicity, hereafter we refer to the photoperiodic treatments as: very early (EEP), early (EP), control (CP), late (LP) and very late (LLP).

Temperature (12.5 °C), humidity (70-90 %) and light sources (six lamps, Philips TL mini 8W/33T, 640 white) were kept the same in all incubators. Loggers (Thermochron iButton, HOBO pendant light logger and TESTO humidity loggers) were used to monitor conditions in the climate cabinets. Larvae were reared individually and fresh oak leaves were provided every other day. Branches were collected from oak trees in Heteren (51°57'21.10»N, 5°44'36.28"E) and screened for presence of other insects. Similar amount of leaves was given to each individual larva. During the experiment, the age of the leaves followed the natural progression of the season.

Larvae were weighed three weeks after the start of the experiment, about halfway the development, and checked for pupation three times a week. Larval developmental time, measured as the number of days from egg-hatching till pupation (i.e. first day that the larvae initiated the cocoon formation) was recorded. On the day of pupation, each individual pupa was weighed and transferred to a falcon tube with vermiculite (sterile artificial soil). All pupae were placed in a single climate cabinet, kept in darkness and exposed to a temperature regime that mimicked natural soil temperatures at 10 cm depth (monthly averages) until adult eclosion. From early November till mid-January pupae were scored for adult eclosion daily.

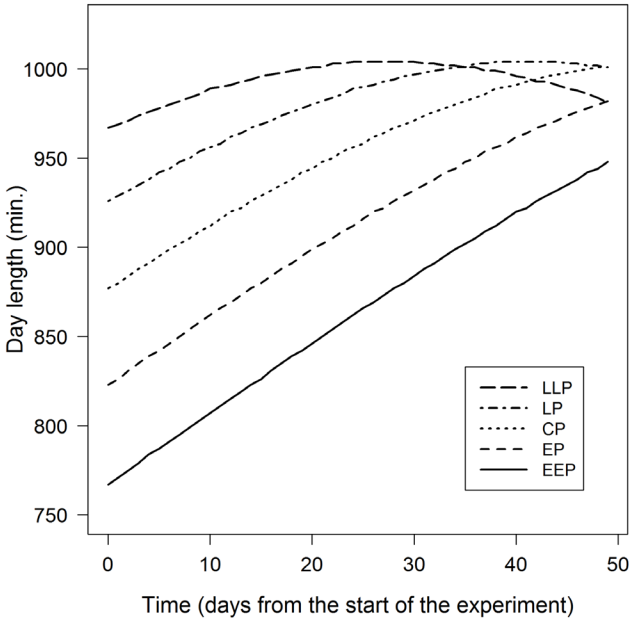

Figure 3 Photoperiodic treatments used in the experiment. All five treatments mimicked the same natural progression of day length but differed on the starting date: 1st April (very early photoperiod, EEP); 15th April (early photoperiod, EP); 1st May (control photoperiod, CP); 15th May (late photoperiod, LP) and 1st June (very late photoperiod, LLP). The experiment started on the 1st May (day 0).

As the adults emerged, date and weight was recorded. Pupal developmental time was scored as the number of days from first day of pupation till adult eclosion. Adult moths were mated within their experimental group (i.e. same photoperiodic treatment) following a half-sib design: two unrelated females were mated with a single male. During mating each female was provided with a piece of rolled paper to lay eggs on. The male was switched from one female to the other every day for a total of four days. In the period from eclosion until the end of egg laying all adults were kept in the same climate cabinet in constant darkness at 10°C. Females were removed five days after mating had started.

The eggs laid were split in sub-clutches, randomly allocated to three temperature treatments and placed at 6.5, 10, or 12.5°C in climate cabinets. Eggs were kept in darkness until egg-hatching. The size of each sub-clutch was approximately 25 eggs; the number of replicas per clutch per treatment depended on the size of the initial clutch laid, ranging between 1 and 10 replicas per temperature treatment.

In all the temperature treatments, eggs were monitored for hatching three times a week and median egg-hatching date was calculated as the date at which 50% of the sub-clutch hatched. Egg developmental time of each sub-clutch was recorded as the number of days from the start of the temperature treatment till egg-hatching. This design, with different temperature treatments during egg development, allowed testing the presence of an interaction between the (larval) photoperiodic treatments and the temperature-depended egg-hatching (i.e. to test for effects of treatment on the reaction norm slopes of eggs hatching versus temperature).

Statistical analysis

Larva, pupal and egg developmental time were individually analyzed using a *coxph* survival model to test for differences among groups of individuals exposed the photoperiodic treatments as larvae. Photoperiodic treatment was fitted as fixed effect in all analyses. In the analysis of egg developmental time we added temperature treatment as a fixed effect. Within each life-cycle stage, we used an ordered heterogeneity test (hereafter referred to as OH test) (Rice *et al.* 1994) to test whether the differences in developmental time among photoperiodic (larval) treatments, followed the predicted direction. This test uses the *p*-value obtained from the survival analysis (non-directional test) and the Spearman's correlation coefficient (*rho*) to test the probability for a trend across the treatments in the predicted direction. All OH tests are one-tailed as we had a directional prediction of the effect of the treatments. Developmental time in all life-stages was tested for the following order: developmental time was expected to be the longest when larvae were exposed to the EEP treatment and shortest when larvae were exposed to the LLP treatment. Overall the developmental time in each treatment was expected to follow the order: EEP≥ EP ≥ CP ≥ LP ≥ LLP.

Differences in larval, pupal and adult weights among experimental treatments were analyzed using an OH test and model estimates were obtained from an analysis of variance (ANOVA) with treatment as fixed effect. The order tested was the same as in the previous analysis: we expected the higher weight in individuals exposed to the EEP treatment and lowest weight in individuals exposed to the LLP treatment. To analyze differences in temperature, humidity and light intensity data during the larval development we also used an OH test and model estimates were obtained from an ANOVA. Differences among groups in the number of individuals that pupated, eclosed and the sex ratio of adults were analyzed with Chi–Square test. All analyses were performed using R software (version. 3.0.2).

Results

Photoperiodic treatments experienced during larval development had no systematic effect on larval developmental time (OH test; p=0.5; Figure 4a, n=443) but did significantly influence pupal developmental time (OH test; p<0.001; Figure 4b, n=254 (153 females and 101 males)) and egg developmental time of the F_1 generation (OH test; p<0.001; Figure 4c at 10° C; see figure S1 in Supplementary Materials for the 6.5 and 12.5 °C temperature treatments).

Effect of the photoperiodic treatment on larval developmental time and performance

Significant differences in larval developmental time between treatments (p<0.001; Likelihood Ratio Test=57.7; Table 1) were not ordered along the expected treatment effects (EEP≥ EP≥ CP≥ LP ≥ LLP). Photoperiodic treatment also did not have a systematic effect on larval weight (OH test; p>0.1) although there were differences among treatments (ANOVA; df=4; p<0.001; Figure S2 in Supplementary Materials). Additionally, no differences among treatments in the number of larvae pupated (X-square=0.916; df=4; p=0.922). All three variables measured in the climate cabinets during larval development, daily mean temperature, humidity and light intensity, differed among treatments (one cabinet per treatment; Figure S3 in Supplementary Materials; ANOVA; p<0.001). However, none of them had differences correlated with the expected treatment order (all OH test; p>0.05).

Effect of the photoperiodic treatment on pupal performance and timing of adult eclosion

Pupal developmental time significantly differed between treatments in the OH test (p<0.001; Table 2). Pupal developmental time was longer in animals that during larval development were exposed to EEP and EP treatments compared to pupal developmental time of animals exposed to LP and LLP treatments. The photoperiodic treatment had an effect on pupal weight at the first day of pupation (OH test; p=0.02; Figure S3 in Supplementary Materials). Pupal weight of larvae exposed to the EEP treatment was the lowest, while pupal weight of larvae exposed to the LLP treatment was the highest. However, differences in weight were not present anymore after pupae completed their development and eclosed as adults (OH test; p>0.1; Figure S4 in Supplementary Materials). We found no differences between the five photoperiodic treatments in the numbers of adults emerged (X-square=3.165; p=0.53; df=4), neither in the sex-ratio of adults eclosed (X-square=0.916; p=0.922; df=4).

Table 1 Analysis of larval, pupal and egg developmental time for the five photoperiodic treatments. Hazard ratio estimates, likehood ratio test and *p*-values were obtained with *coxph* survival model per each life-cycle stage separately. The order heterogeneity test ($p^{(b)}$) tested the directional hypothesis that duration of each stage was affected by the photoperiodic treatments in the following order: EEP≥ EP≥CP≥LP≥LLP (see Materials and methods section for more details on the photoperiodic treatments). Egg clutches of the F_1 were split over three temperature treatments (6.5, 10 and 12.5 °C). Effects of temperature and photoperiodic treatment were tested in the same model.

	proportional hazard model estimates								**Likelihood ratio test**	$p^{(a)}$	***rho***	$p^{(b)}$
	photoperiodic treatment (*)					**temperature** (**)						
	EEP	***EP***	***CP***	***LP***	***LLP***	***6.5°C***	***10°C***	***12.5°C***				
larval developmental time	0.46	-0.68	0	-0.33	-0.36				57.7	<0.001	0	0.5
pupal developmental time	-1.14	-0.78	0	0.6	1.5				154	<0.001	1	<0.001
egg developmental time (F_1)	0.92	0.42	0	-0.16	-0.52	0	6.97	10.99	866	<0.001	-1	<0.001

(a) *p*- values are for the *coxph* survival function ; (b) *p*- values are for the ordered heterogeneity test

(*) proportional hazard estimates for the effect of photoperiodic schedule are calculated against the control group (CP)

(**) proportional hazard estimates for the effect of temperature are calculated against the 6.5°C treatment

p- values for the *coxph* function relative to the temperature treatment were all significant (p <0.001, not shown in the table)

The interaction (temperature*photoperiodic treatment) was not significant

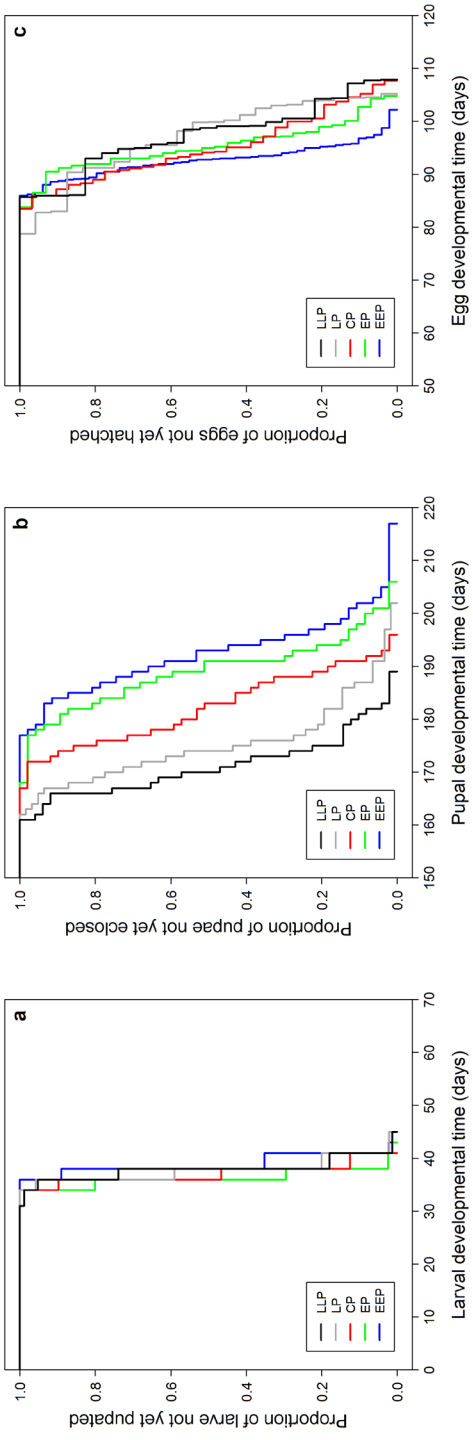

Figure 4 Developmental time of winter moth life-cycle stages: a) larval; b) pupal and c) F_1 egg developmental time. Colors mark the five photoperiodic treatments. The photoperiodic treatment affected pupal and egg developmental time but not larval developmental time (see table 1 for statistics). Eggs were kept at 10 °C throughout development (see Supplementary materials for the 6.5 and 12.5 °C treatments).

Effect of the photoperiodic treatment on timing of egg-hatching of the F1 generation

The photoperiodic treatments experienced during larval development had a significant effect on egg developmental time of the following generation (OH test; $p<0.001$; Table 1). Eggs laid by adults exposed to the early larval treatments (EEP and EP) had shorter developmental time than eggs laid by adults from the late larval treatments (LP and LLP). The effect of temperature on subsequent egg developmental time was significant, but this temperature effect did not depend on preceding photoperiod treatments (interaction temperature*treatment was not significant; ANOVA $p>0.1$).

Discussion

In the winter moth the phenological match between egg-hatching and bud bursting of its host plant is crucial for survival. Our long-term data (1994-2014) show that egg-hatching phenology strongly varies from year to year but timing of adult eclosion shows much smaller inter-annual variation than egg-hatching (Figure 2b). In fact, in our study sites, in the past twenty years the peak of adult eclosion has occurred every year around the same week, with a variation limited to 6.5 days from a fixed calendar date. Therefore, the variation cumulated during egg-development is not fully carried over to the following adult stage, although some variation in egg-hatching phenology exists. This lack of carry over effects between years seems an evolutionary optimal strategy since at adult eclosion temperature pattern for the next spring is not predictable while the date at which the eggs are laid affects the date of hatching (Figure 2a). As a result, carry-over effects at egg-hatching are offset during the following stages and do not affect the timing of egg-laying and thus the egg-hatching of the next generation.

Our experimental findings show that winter moth larvae use photoperiodic cues to regulate their following pupal developmental time and, as a consequence, offset the phenological carry-over effects of timing of egg-hatching. In fact, the manipulation of the photoperiod experienced during larval development affected the pupal developmental time and, in turn, timing of adult eclosion. Developing larvae exposed to late photoperiodic treatments had an earlier adult eclosion date than larvae exposed to early photoperiodic treatments. Overall, these results suggest that photoperiod regulates winter moth phenology via maintaining the synchrony between a fixed calendar date and adult eclosion, which subsequently influences egg-hatching phenology in the next spring. Moreover, the photoperiodic response increases the within-year synchronization among individuals originating from early- and late-hatching eggs. In the winter, at low temperatures, the chance to find a mate and reproduce drastically increases when the adults eclose at the same time.

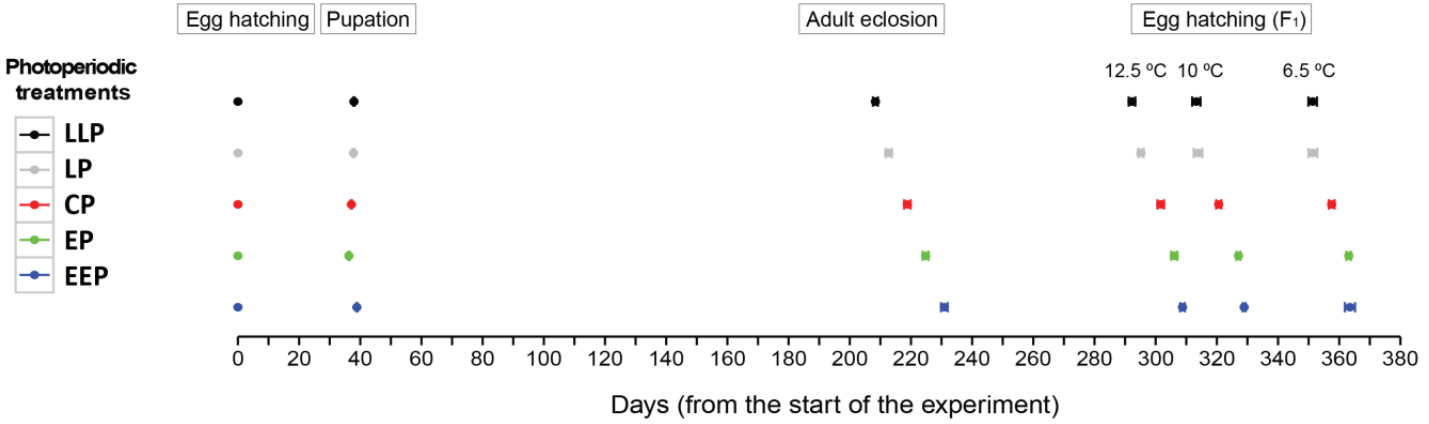

Figure 5 Annual cycle of the experimental animals for each photoperiodic treatment. Winter moths were exposed to the photoperiodic treatments only during larval development. Note that after pupation, individuals from different treatments were exposed to the same conditions. Eggs laid by the experimental animals were kept at 12.5, 10 and 6.5 °C. Points represent mean duration of each stage per experimental group, standard error bars are indicated except for the start of the experiment at day 0.

The perceived photoperiod experienced as larvae also influenced egg developmental time of the F_1 generation, but in the opposite pattern than the one observed for the pupal development: adult parents originating from larvae exposed to late photoperiods, laid eggs that had longer developmental time compared to eggs laid by parents originating from larvae exposed to early photoperiods. However, the differences among treatments in developmental time of the F_1 eggs were less than the differences in adult eclosion between the parents. Therefore, the overall developmental time between the egg-hatching of the parents and the egg-hatching of the F_1 was longest in animals exposed as larvae to early photoperiodic treatments. One possible explanation to the opposite pattern found in the egg developmental time is a maternal effect. As adult, females experienced their early (or late) timing of egg-hatching as being successful (i.e. food quality was kept high throughout the larval rearing). Therefore the females might have varied the composition of the eggs laid in order to make the eggs hatch early (or late) and thus enhancing adaptation of their offspring to the phenology of their host tree. This is similar to what was found in the same species when the timing of egg-hatching relative to the bud opening of the host plant was manipulated (van Asch *et al.* 2010).

Finally, our results clearly show that winter moths are able to perceive photoperiodic cues and respond to them. This also implicates the presence of a photoperiodic timing system able to measure day length. Additionally, this study also provides the evidence that the period during which the winter moth is sensitive to photoperiod is during larval development. From this study, however, it is not possible to infer the mechanisms underlying the observed photoperiodically induced phenological response. Behaviour, physiology and molecular pathways involved in photoperiodic measurement and photoperiodic response need further investigations.

Due to our experimental design (i.e. one climate cabinet per photoperiodic treatment), there were differences among the climate cabinets during larval development. In particular, the differences in temperature (range 12.5-13.2 °C; Figure S3a in Supplementary materials), biologically relevant to the rate of larval developmental, were likely to influence larval developmental time. Therefore, in the analysis of larval developmental time we cannot disentangle between the effect of the photoperiodic treatments and temperature. The differences in the other variables were comprised within a smaller biological meaningful range (relative humidity 80-90% in Figure S2b; light intensity 400-500 lux in Figure S2c). In the analysis of the pupal and egg (F_1) developmental time, the differences, in none of the variables, were ordered along the photoperiodic treatments, therefore it is unlikely that they influenced the patterns found in the developmental time of subsequent life-cycle stages. Weight of individuals at different life-stages was not affected by the photoperiodic schedule with exception of pupal weight. Larvae experiencing the latest photoperiodic schedule pupated at heaviest weight. However, as weight was

measured on the first day of pupation, the differences observed could also be due to the temperature differences among cabinets during larval development.

Photoperiodic responses in insects represent highly evolved adaptations to daily and seasonal changes in biotic and abiotic environment. They have been extensively studied, particularly with respect to the processes of initiation and termination of diapause. In most diapausing species two distinct stages can be identified: a "sensitive stage" (i.e. a period during which the cue is perceived) and a "responsive stage" (i.e. when the response to the cue takes place). These two stages can be largely separated and the response to a given *stimulus* can occur one, two or more generations after the cue is perceived ((Tauber *et al.* 1986) and references therein). In the winter moth system this seems also to be the case, with the larval stage being sensitive to photoperiodic cues and the pupal stage being the responsive stage. However the winter moth does not initiate a clear go/no-go response typical of most diapausing species. In this insect there is no evidence of diapause at any stage and our findings suggest, rather than a clear photoperiodically–induced diapause response, the presence of a quiescent phase that is finely modulated by photoperiodic information. The perceived photoperiod seems to be used to regulate the duration of pupal development (perhaps by resetting the annual endogenous clock) and as result, maintain the adult eclosion at a fixed calendar date. In a climate change scenario this mechanism, which is still not yet accurately described and therefore requires further studies, could be useful in maintaining an appropriate phenology throughout the entire life-cycle in the winter moth and likely, in other lepidopteran species.

Acknowledgements

We are thankful to Family Van Boetzelaer van Oosterhout for kind permission to collect animals in their woodland. We are grateful to B. van Lith, L. Bakker, R. van Dongen, A. de Plaa and O. Esbri Senar for help during the setting up of the experiment. Phillip Gienapp gave valuable comments regarding the statistical analysis. The project was supported by a NWO-VICI grant to M.E. Visser.

Appendix to chapter 5

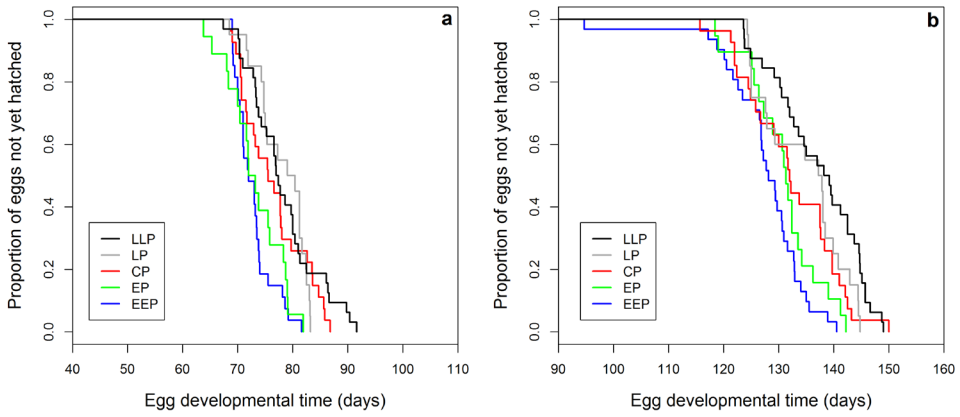

Figure S1 Developmental time of eggs (F_1 generation) kept at a) 12.5 °C and b) 6.5 °C. Colors indicate the photoperiodic treatments that the parents experienced during larval development. Eggs laid by moths exposed to very early photoperiod (EEP) had the shortest developmental time (see Table 1 for statistics).

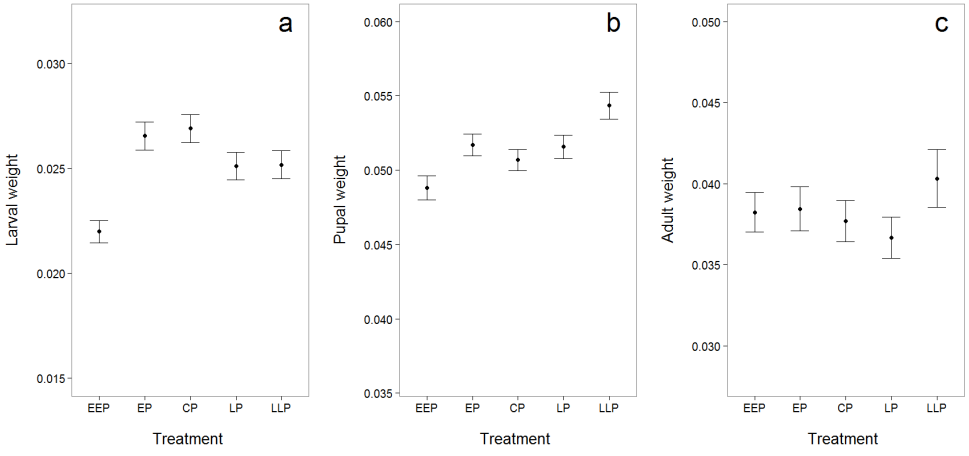

Figure S2 Mean a) larval; b) pupal and c) adult weight for the five photoperiodic treatments. Photoperiodic treatments did not affect larval weight (OH test p>0.1). Mean pupal weight at first day of pupation was significantly affected by the photoperiodic treatment (OH test p=0.02). Differences in pupal weight among treatments were likely to be due to the photoperiodic treatments; however, the pattern found could also be influenced by the temperature during larval development. Mean adult weight at the day of eclosion was not affected by the photoperiodic treatments (OH test p>0.1). Bars represent standard error.

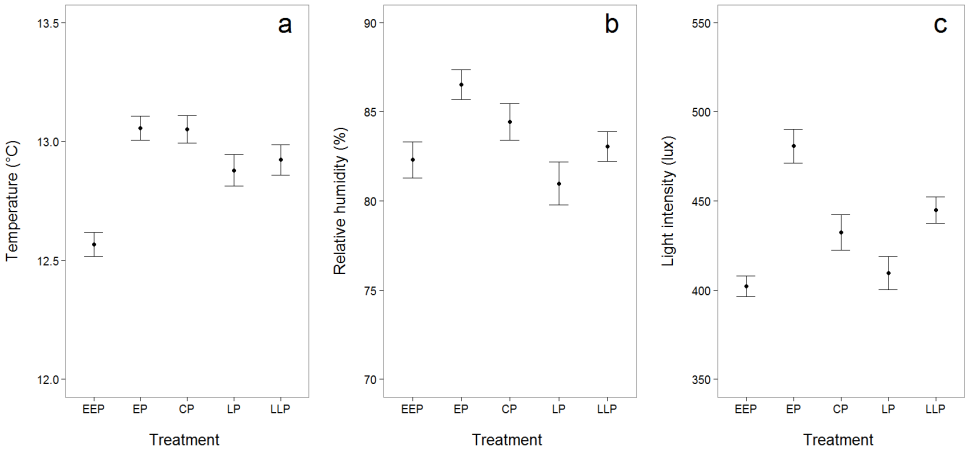

Figure S3 Mean a) temperature; b) relative humidity and c) light intensity (±SE) for the five photoperiodic treatments. Each treatment was associated with one climate cabinet. Bars represent the standard error. All the three variables differed among treatments (ANOVA $p<0.001$; df=4) but in none of them the differences were ordered along the photoperiodic treatments (OH test $p>0.05$).